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Departamento de Biologia

Filipa Isabel de Barros Maia

Estudo do efeito de pós de vidro de óxido de titânio in vitro e in vivo

Study of the effect of titanium oxide glass powder *in vitro* and *in vivo*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Maria Helena Fernandes, Professora Associada do Departamento de Engenharia Cerâmica e do vidro da Universidade de Aveiro.

Dedico este trabalho à minha família.

o júri

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palavras-chave Pó de vidro, TiO_2, reactividade, células MG-63, peixe zebra, embrião

resumo

No presente trabalho propõe-se estudar o efeito dos produtos de dissolução de pós de vidro com dióxido de titânio (14.48 mol% TiO_2), óxido fosfórico (42.76 mol% $P_2O_{5)}$ e óxido de cálcio (42.76 mol% CaO) (extracto).
O sistema vítreo testado ($14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$, o qual foi formulado por Silva et al., 2008) é um cerâmico à base de cálcio e fosfato (CPC) e o principal desafio consiste na sua aplicação em reconstrução óssea. CPCs são vantajosos devido à ausência de toxicidade e resposta inflamatória e à capacidade em ligar ao tecido do hospedeiro.
A incorporação de TiO_2 no vidro aumenta a durabilidade e a estabilidade química do sistema, a qual permanece pouco clara. Outras características importantes são a boa bioactividade *in vitro* quando imerso em soluções plasmáticas e a nucleação da apatite na superfície do material (Silva et al., 2008).

As exposições realizaram-se numa linha de células semelhantes a osteoblastos e resistente (células MG-63) e em embriões de peixe Zebra (*Danio rerio*) como organismos alvo. Esta abordagem é uma perspectiva inovadora de testes de citotoxicidade usando modelos *in vitro* e *in vivo*.

Caracterizou-se o extracto (concentração iónica e pH) após a dissolução do pó em PBS (*Phosphate Buffered Saline*) e em água do peixe Zebra. As diluições de extracto estudadas foram entre 0.1% e 50%.

Os testes *in vitro* consistiram em ensaios de viabilidade e proliferação celular (MTT), morfologia das células e colónias (CLSM) e RT-PCR com *primers* específicos implicados na remodelação do osso. Os dados de MTT foram analisados através de comparações hierárquicas usando *nested* ANOVA.

Em relação às experiências *in vivo* foram seleccionados embriões viáveis e foram realizados ensaios de toxicidade durante o desenvolvimento embrionário. Após a exposição, a concentração dos iões foi medida via ICP. Congelaram-se as larvas para testar os biomarcadores (AChE, LDH e GST), para averiguar possíveis efeitos em actividades enzimáticas. Usou-se *One-way* ANOVA para tratar os dados.

O extracto causou um efeito inibitório dependente da concentração na viabilidade/proliferação das células MG-63 expostas a 20%-50% de extracto e é evidente entre os dias 3 e 7. Contudo, as células testadas foram capazes de recuperar. Foi detectado um aumento significativo na taxa de crescimento celular entre os dias 7 e 10. A morfologia celular e a organização do citoesqueleto de F-actina (CLSM) não foram afectadas, apesar de ter ocorrido uma redução no número de células imersas em 50% de extracto. O tempo dos eventos celulares pode ser apropriado para ocorrer a reconstrução do osso. A expressão dos genes ALP e BMP-2 foi estimulada (envolvidos na formação do osso) e M-CSF e RANKL foi *down-regulated* (relacionados com a digestão do osso).

Os estudos *in vivo* (Teste 2) mostraram que a exposição a 10% e 20% de extracto promoveram stress químico nos organismos peixe Zebra seleccionados, e não usaram a via GST para possível eliminação de tóxicos. Os resultados do teste 1 revelaram efeitos possíveis do extracto como a disfunção no sistema nervoso em todos os tratamentos (AChE), a ocorrência de condição de stress químico para ~2.5% de extracto (LDH) e efeito negativo na actividade da GST para ~10% e 20% de extracto (os organismos seleccionaram outras vias de desintoxicação quando expostos a concentrações maiores de extracto ou este enzima foi desactivado, danificado ou inibido). A interacção entre os iões analisados e os embriões/larvas de peixe Zebra e o seu efeito subsequente podem estar relacionados com a forma química dos produtos libertados do biomaterial teste. Por exemplo, a quantidade de iões Ti, Ca e P foi superior após 168 horas que 336 horas de incubação, sugerindo a precipitação de partículas ou presença noutra forma não detectada pelo ICP.

Os modos de acção envolvidos são pouco claros e mais informação é necessária para contar a história deste bioprocesso – reconstrução do osso. O vidro testado parece ser promissor em regeneração óssea.

keywords Powder glass, TiO_2, reactivity, MG-63 cells, zebra fish, embryo

abstract

In the present work it is proposed to study the effect of the dissolution products of powders glass with titanium dioxide (14.48 mol% TiO_2), phosphorus oxide (42.76 mol% P_2O_5) and calcium oxide (42.76 mol% CaO) (extract).
The glass system tested ($14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$, which was formulated by Silva et al., 2008) is one Calcium Phosphate Based Ceramic (CPC) and the principal challenge is its application in bone reconstruction. CPCs are advantageous because the absence of toxicity and inflammatory response and the ability to bond to host tissue.
The incorporation of TiO_2 in glass increases the durability and chemical stability of system, which is unclear. Others important characteristics are its good bioactivity *in vitro* when immersed into plasmatic solutions and nucleation of apatite on the material surface (Silva et al., 2008).
The expositions were realized using an osteoblasts-like resistant cell line (MG-63 cells) and zebrafish (*Danio rerio*) embryos as target organisms. This approach is an innovative perspective of cytotoxicity tests using *in vitro* and *in vivo* models.

The extract was characterized (ionic concentration and pH) after dissolution of the powder in PBS (Phosphate buffered Saline) and in Zebrafish water. Dilutions of extract studied were between 0.1% and 50%.

In vitro tests consisted of viability and cell proliferation assays (MTT), cells and colonies morphology (CLSM) and RT-PCR with specific primers involved in bone remodeling. MTT data was analyzed with hierarchical comparisons using nested ANOVA.

For the *in vivo* experiments viable embryos were selected and toxicity assays were performed during embryonic development. After exposure, ions concentration was measured by ICP. Larvae were frozen to test biomarkers (AChE, LDH and GST) to investigate possible effects on enzymatic activities. One way-ANOVA was done to treat data.
The extract caused a slight dose-dependent inhibitory effect in viability/proliferation of MG-63 cells exposed to 20%-50% of extract and it is evident between 3 and 7 days. However, cells tested were able to recover. It was detected a significant increase in CGR between 7 and 10 days. Cell morphology and the organization of F-actin cytosqueleton (CSLM) weren't affected, besides there was occurred a reduction in cell number of cells immersed in 50% of extract.

The time of cells events can be appropriate for bone reconstruction occur. ALP and BMP-2 genes expression were stimulated (involved in bone formation) and M-CSF and RANKL were down-regulated (related to bone resorption).

The *in vivo* studies (Test 2) showed that the exposition to 10% and 20% of extract promoted chemical stress in Zebrafish organisms selected, and they didn't use GST pathway for possible toxics elimination. Results of test 1 revealed possible effects of extract like disruption of the nervous system in all treatments (AChE), occurrence of chemical stress condition for ~2.5% of extract (LDH) and negative effect on GST activity for ~10% and 20% of extract (organism selected other pathway of detoxification when exposed to higher concentrations of extract or this enzyme was deactivated, damaged or inhibited). The interaction between ions analyzed and Zebrafish embryos/larvae and its subsequently effect can be related to chemical form of products released from biomaterial tested. For example, the amount of Ti, Ca and P ions was higher at 168 hours of incubation than 336 hours, suggesting precipitation of particles or presence in other form not detected by ICP.
The ways of action involved are unclear and more data is required to tell the history of this bioprocess – reconstruction of bone. The glass tested seems to be promissory in bone regeneration..

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1. INTRODUCTION

1.1. BONE TISSUE DYNAMIC

Bone tissue is able to repair small defects, caused by trauma, disease or developmental anomalies (Neel et al., 2008). The remodelling process begins in foetal life and evaluates to adult stage, occurring in units comprising osteoblasts, osteoclasts, osteocytes and cells precursors. Osteoclasts, giant multinucleated cells, are presented on bone surfaces and remove old bone tissues- Osteoblasts**,** cells originated from less differentiated precursor cells and found in the growing portions of bone, refill the resorption cavities by the production of the extracelular bone matrix and its own mineralization. Extracellular matrix is composed by calcium and other minerals, which give to tissue the rigidity, strength and some elasticity, and by collagen type I and other proteins. The osteocytes are responsible for maintaining the mineral composition of matrix.

There is a balance between bone resorption and bone formation to achieve homeostasis of tissue. Its disequilibrium can lead to diseases, for example osteoporosis (bone less), which can lead to fracture of bone, or osteopetrosis (bones become denser) (Pekkinen, 2008). Osteoporosis is the most common skeletal disease and increases with aging. The decrease in bone mass and the changes in micro-architecture increase the bone fragility and the risk of fracture (Augat et al., 2010). As consequence, many patients have poorer quality of life, without independence. Curiously, many diseases and conditions can increase the predisposition of persons to have osteoporosis and secondary vertebral compression fracture, for example the advanced age, diabetes mellitus, vitamin D deficiency, menopause and tumor (Cotton et al., 1998).

In bone activity five stages can be distinguished: osteoclastic resorption, reversal phase, preosteoblastic migration and differentiation into osteoblasts, osteoblastic matrix function and finally mineralization (Pekkinen, 2008). The duration is more or less 4-6 weeks (two weeks to resorption and several months to formation).

Osteoblasts can acquire **two main functional states**. On the first, they emit important extracellular signals (cytokines, growth factors, ALP, Col-1, Cbfa 1 and OCN) for specific

genes expression, which are implicated in osteoblasts differentiation, development and formation functions. In the second one, they control osteoclasts activities during normal turnover of bone, either differentiation or function (through a specific receptor-ligand interaction, the RANK-RANKL connection) (Sun et al., 2009).

1.2. REGENERATIVE MEDICINE

Human body is poor at repair. In contrast, some animals are able to regenerate own tissues; for example amphibians can produce a new limb in 70 days. This way, **Regenerative Medicine** has been useful which concern on repair of large bone defects (Neel et al., 2008) and was probably firstly described by William Heseltine (Chairman and CEO of Human Genome Sciences (Rockville, MD, USA)), when he defined Regenerative Medicine as 'the broad range of disciplines adopted by companies working towards a common goal of replacing or repairing damaged or diseased tissue' (Kemp, 2006).

First strategies were replacements of the injured organs by mature fully functional ones since 1950s (with identical twins and the chirurgic intervention was successful). Organs transplant evolved when the immunosuppression was understood. After 1960s scientist became to understand cell biology, manipulating fragments of tissue alive from the surface of body and purifying different cell types samples to culture single-cell monolayer, and they acquired a higher vision about 'organotypic cultures'.

The highest point was the cloning of the sheep Dolly and the isolation of human embryonic stem cells, in the end of 1990s (Kemp, 2006).

Tissue Engineering' (subset of regeneration medicine) studies has began in four independent laboratories at the Massachusetts Institute of Technology (MIT). Nowadays there are many enterprises and labs which developed own strategies to produce and sell innovative products such as tissue-repair cells (autologous chondrocytes, autologous keratinocytes and bone marrow stem cells) and the development of acellular scaffolds which induced host' cells to reconstruct the tissue target (generate by Yannas' laboratory) (Kemp, 2006).

The Regenerative Medicine is a multidisciplinary field, due to cooperation of tissue engineering, stem cell therapy, regenerative factors, biomaterials and therapeutic cloning (Kemp, 2006).

Biomaterials are substances used for total or partial replace of biological systems. The period of exposure depends on its application and durability. They should be biocompatible (biologically accepted by the organism, without any kind of injury effect when in contact with tissues or physiological fluids) and functional according to the initial design. Metals and alloys, ceramics and glasses, polymers and composites are examples of Biomaterials (Silva et al., 2008). Synthetic materials are advantageous because they are available in a wide range of sizes and shapes, can have several applications, are easily sterilized, do not represent a problem concerning ethnical issues, and have reasonable costs.

Calcium phosphate based ceramics have been highlighted due to the absence of toxicity and inflammatory response and because they are able to bond to host tissue, the main requirement for the so called bioactivity. The definition of Bioactivity (Etymology: GK, *bios* is life, and L, *activus* is with energy) differs according to the area applied. It is attributed to the effect of a given agent upon a living organism or tissue[1].

Hench (1971) created the first bioactive material (the Bioglass®45S5) for orthopaedic applications, a material not rejected by the organism and responsible for the decrease of the post-surgery period. The Bioglass has been widely studied, which quickly adhere to bone tissue by the formation of an apatite superficial layer (base of bioactivity) (Silva et al., 2008).

The chemical stability of calcium phosphate-based glasses is not entirely understood and the majority of glass systems are too soluble. Some strategies have been developed, such as the application of heat treatments to the base glass, which allows the precipitation of

[1] http://www.thefreedictionary.com/bioactivity (3/09/2010, at 17:15 hours).
http://www.google.pt/search?hl=pt-PT&defl=en&q=define:Bioactivity&sa=X&ei=tByBTMjfM4HNjAeKjsHxAQ&ved=0CBUQkAE (3/09/2010, at 17:10 hours).
http://medical-dictionary.thefreedictionary.com/bioactive (3/09/2010, at 17:05 hours).

crystals inside the glassy matrix, or the modification of the chemical composition by adding small amounts of other oxides. Titanium dioxide has been incorporated into glass systems, based on evidences that titanium exhibited good bioactivity *in vitro* (formation of Ti-OH groups) when immersed into plasmatic solutions and nucleation of apatite on the material surface). Addition of titanium oxide to a glass composition can improve the chemical durability and the stabilization of the glass system (Silva et al., 2008).

With the progress in the development of prosthetic devices, adequate strategies have been defined for testing biofunctionality and biocompatibility of the devices, which shouldn't influence negatively the organism.

1.3. TOXICOLOGY ASSAYS

Toxicology and Biomaterials are related, because it is crucial accessing to organism' answer to biomaterials' exposure. The devices shouldn't be toxic. For instance, substances can migrate from the biomaterial (if it degrades) and damage the surrounding tissues. However, systems can be specifically designed to be toxic and release drugs in target points ("smart bomb" drugs are examples of release systems created to kill cancer cells) (Ratner et al., 1996).

Actually there is a need to restring experimental procedures in animal models to standard procedures good established in laboratories, due to ethical problem. The challenge is building the picture of pathways involved by compilation of information of assays, recurring *in vitro* systems and some promissory *in vivo* models (more complex). With *in vivo* systems it is possible to highlight the effect of biomaterials and exclude the inappropriate materials for man (first screening). The biomaterial can be exposed directly (cells are seeded on contact with material) or indirectly (cells are exposed to extract of material), where cytocompatibility and cytotoxicity assays are analyzed, respectively (Figure 1) (Silva et al., 2005).

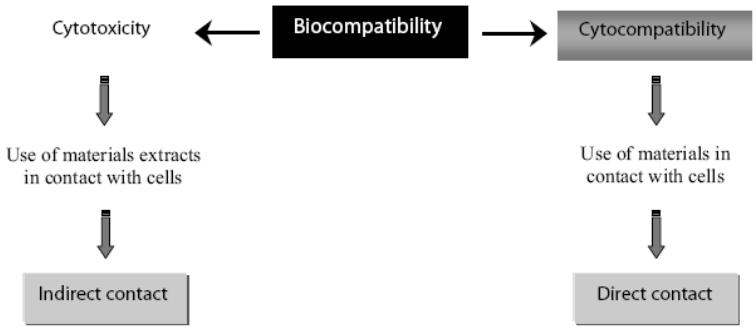

Figure 1: Biocompatibility, cytotoxicity and cytocompatibility in *in vitro* tests (Silva et al., 2005).

Usually, scientific experiments are performed with two kinds of cells, primary cell lines (ideally) or cell lines (routinely used in laboratories), and sometimes just one first screening with cell lines is made and further analyzes on adequate primary cultures because these cultures contain a variety of kinds of cells, so it is more realistic and represent the happenings on tissue (Figure 2) (Kemp, 2006).

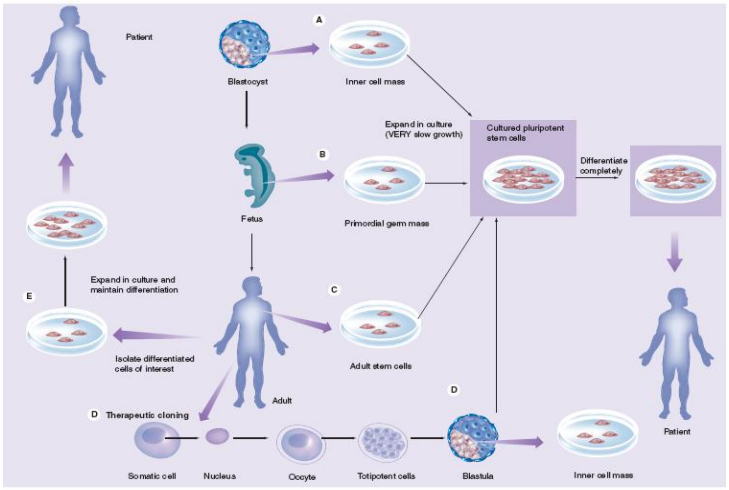

Figure 2: Schematic representation of cell's sources (Kemp, 2006).

Cell lines are transformed primary cells and they are previously established in laboratory. They are immortal, can come from different species and tissues and appear with morphological alterations (such as decrease cell size, reduced adherence and higher nucleus). Primary cell lines are detached from one piece of tissue or are migrated spontaneously from one explant, and can be propagated as an adherent monolayer or

cellular suspension. Besides ability in proliferate and replicate, functions of specialized cells can be lost with culture propagation (Silva et al., 2005).

The degradation profiles of the proposed product should be established into solutions similar to body fluid, to simulate its behavior in contact with the body. So, extracts from materials and specific medium culture have been added to culture.

In cytotoxicity screening tests the morphology of the system studied influences its behavior. When the surface area is larger more ions dissolve into the environment, due to greater degradation rate. Examples of cell manifestation are death (endpoint), loss of membrane integrity, reduction on cell adhesion and spreading, altered cell morphology and reduction in biosynthetic activity. To explore these features some techniques can be used such as morphological, biochemical and genetic (Silva et al., 2005).

In the present study, the material tested is a promissory bone regenerator-glass containing titanium oxide with the following formulation (14.48 mol% TiO_2, 42.76 mol% P_2O_5 and 42.76 mol% CaO) $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ (Silva et al., 2008). The models used to access the behavior of the material studied are MG-63 cells cultures, an osteoblastic resistant cell line culture, and Zebrafish early-life stages (*Danio rerio*).

2. STATE OF THE ART

2.1. EXAMPLES OF DESIGNED BIOACTIVE MATERIALS

Nowadays, **bioactive materials** have been applied in several bone repair situations, because they are able to chemically bond to bone, causing precipitation of calcium phosphate when in contact with body fluids and becoming part of the surrounding bone (Piskounova et al., 2009).

Examples of bioactive materials are Hydroxyapatite (HA) and bioactive inorganic glasses, which generate a layer of crystalline hydroxyapatite [$Ca_5(PO_4)_3OH$], allowing to interact with bone and resist to mechanical forces. Together with Bioglass® (silica based glasses), and calcium phosphate hydroxyapatite (HA), crystalline titanium dioxide (TiO_2) of rutile or anatase (Piskounova et al., 2009), and Zirmonium oxide (ZrO_2C) (Sollazo et al., 2007) are also used for their bone bonding capability.

The phosphate-based glasses emerged as an alternative to silica-based ones and appeared with broad spectrum of hard tissue engineering applications, because its components are natural constituents of the human body (Neel et al., 2008).

According to Brauer et al. (2006) the incorporation of TiO_2 into the glass system P_2O_5CaO-MgO-Na_2O decreased the solubility of glass in water and SBF (Simulated Body Fluid), but SiO_2 addition produced a higher dissolution rate compared to the original glass). The proliferation of cells tested (MC3T3-E1.4 murine preosteoblast cell line) increased with the decrease of the glass solubility (Brauer et al., 2006). These results showed that cell responses could be modulated by changing the solubility of the glasses.

Piskounova et al. (2009) developed a bioactive crystalline titanium dioxide (TiO_2)/hydroxyapatite (HA) surfaces combined with BMP-2 (bone morphogenetic protein 2), by a soaking method, and studied its potential role as implant coatings to improve osseointegration.TiO_2, is bioactive and assured long-term fixation of the implant and the outer layer improved the initial bone attachment. The results showed that the crystalline TiO_2 and the HA surfaces were more favorable than the native titanium oxide surface, with respect to cell viability, morphology and initial differentiation.

Zirconium oxide coatings (ZrO_2C) have been used to produce prosthetic devices and showed to alter osteoblast activity, promoting bone formation. Sollazzo et al. (2007) identified genes

involved on regulation of osteoblast-like cell line (MG-63) exposed to ZrO_2C by microarray technique (the microarray slides contained 20,000 genes). The results reveal that this coating up/down regulate some functional activities of MG-63, such as cell cycle regulation, signal transduction, immunity, and cytoskeleton component (Sollazzo et al., 2007).

2.2. TREATMENT OF BONE FRACTURE

Nowadays there are several studies about the treatment of bone fracture, since the chirurgical implantation of cancellous bone autografts (to stabilize the fracture) or other implants (to replace the damaged bone) such as metallic based to augmentation procedures (such as vertebroplasty and kyphoplasty).

The introduction of the autograft referred allows treating of patients with poor osteogenic potential by bone union. However, the risk of infection is high (Lee et al., 2009) and for instance, in the case of osteoporotic bone fractures, it is required a stable fixation of fracture, due to reduced mechanical support (Pesce et al., 2009; Augat et al., 2010). Conservative therapeutics such as bed rest, analgesic medications and physical therapy, were usually used in osteoporotic bone fracture treatment (Uppin et al., 2003).

When bone tissue engineering emerged, the challenge was the formation of new bone tissue to replace the damaged one. So, researchers combined scaffolds with osteoblasts or osteogenic potential cells. But, the clinical cases reported are yet few (Lee et al., 2009).

Other strategy was the augmentation of the weak bone, by the injection of bone cement (Larsson, 2006). The finality of Vertebroplasty and Kyphoplasty is the treatment of the intense pain caused by vertebral compression fractures (VCF), through the injection of particular cement to fill the fracture under anesthesia and fluorescence guidance or computed tomography (for positioning the needle of syringe). These medical procedures differ in some aspects - the kind of cement and the utilization of a balloon tamp in Kyphoplasty.

In Vertebroplasty the material injected within the vertebra is usually methacrylate (acrylic fusion) and give strength to osteoporotic bone, repairing a non-healing compressive fracture. The fracture identified is filled by material.

In contrast, in Kyphoplasty the hole is firstly created into the area damaged by the insertion of the needle and after the balloon. With the space formed the balloon can be removed and the cement mixture is injected[2] (Cotten et al., 1998).
These procedures decrease the risk of infection, although having some drawbacks. For example, in which concerns to vertebroplasty the acrylic mass injected under pressure can pass into the spinal canal[3].

Nakano et al. (2006) referred that CPC (Calcium Phosphate bone Cement)-assisted vertebroplasty revealed better results (clinical and radiological) than conservative treatment used in primary osteoporotic vertebral compression fractures.
The choice of products or biomaterials injected depends on the kind of lesion and the effect desired, such as bone reconstruction or strengthening, lesion destruction or thrombosis. In bone augmentation strategy Polymethylmethacrylate (PMMA) has been used although its acceptance is still controversial (Larsson, 2006; Kobayashi et al., 2007), and also cements as calcium-phosphate, calcium-sulphate or bioglass (in extremity fractures and VCF) (Larsson, 2006). Bioglass can complement the metal devices for complex fractures and its formulation can supplement antibiotics or osteoconductive proteins (Larsson, 2006).

Finally, some researchers believed that stimulation of bone healing process is the key of fracture repair (Shadmehr et al., 2009; Chao et al., 2003). However, it is needed a deep study about that, because bone repair exhibit several pathways with many combinations (Chao et al., 2003). Shadmehr et al. (2009) developed and studied a mechanical stimulation device that applies continuous and uniform interfragmentary movements into lesion area.

The therapeutic(s) used depend(s) on multiple factors, such as the age of patient, the ability of damaged area for self healing, the kind of bone fracture and the clinical story of patient. It is important to plan the interventions to maximize their beneficial effects.

[2] http://www.radiologyinfo.org/en/info.cfm?pg=vertebro (30/08/2010, at 16:14 hours)
http://emedicine.medscape.com/article/1835633-overview (30/08/2010, at 16:20 hours)

[3] http://emedicine.medscape.com/article/1835633-overview (30/08/2010, at 16:20 hours)

2.2.1. CALCIUM PHOSPHATE MATERIALS

According to some researchers, calcium phosphate bone cements (CPCs) such as BoneSource® have been used and intensively studied for treatment of skeletal defects (Kobayashi et al., 2007), and also for fixation of screw (Taniwaki et al., 2003) or of suture anchors (Oshtory et al., 2010), for example.

BoneSource® gradually transforms into calcium phosphate with low crystalline order (like bone mineral) and without heat emission (Kobayashi et al., 2008).
CPCs can be supplemented with other compounds to increase the viscosity, allowing its injection and local application.
Kobayashi et al. (2008) studied the histological and compressive properties of a combination of CPC and carboxymethyl cellulose (CMC) in a sheep vertebral void model. The CPC is reported as BoneSource and is an equimolar mixture of tetracalcium phosphate and anhydrous dicalcium phosphate. CMC was added to form a gel. This gel solution was injected at L3 and L5 in each spine of 40 sheets to fill the defects (the voids were surgically created). After different periods from surgery (0, 3, 6, 12, 24 and 36 months) some evaluations were made against 35 non-operated animals (control), to identify undecalcified and decalcified areas and new bone formation, empty space, fibrous tissue, and residual cement at area of original defect. The researchers concluded that the cement had excellent characteristics, concerning to biocompatibility, osteoconductivity and adequate compressive strength (the augmentation of bone didn't illustrate significant differences compared to control), and was replaced by bone and bone marrow (underwent steady resorption). Besides the variations observed among animals, the area studied was filled approximately by 14% of bone, 82% of cement and 4% of bone marrow at 36 months (on average).

2.3. TEST OF DIFFERENT COMPOSITIONS OF ONE MATERIAL

Silva et al. (2008) studied the *in vitro* surface reactivity of two glasses from the systems TiO_2-CaO-P_2O_5 and SiO_2-CaO-P_2O_5 with the same molar content of TiO_2 and SiO_2, by the immersion of powder glasses in Simulated Body Fluid (SBF) and kept at 37ºC during different

periods up to 14 days. Different dissolution behavior was detected (by Inductively Coupled Plasma), due to different glass structure (accessed by Raman Spectroscopy). For system with SiO_2 degradation was high and all ions dissolved resulted in the formation of phosphoric acid. As to system with TiO_2 a Ca-P rich layer was formed on glass, due to small consumption of Ca from the synthetic physiological fluid. Ti and P concentrations were stable in the fluid. This test was important to define the most suitable glass compositions, for the proposed applications.

2.4. ABOUT THE MATERIAL TESTED

Silva et al. (2008) studied two approaches with the glass-test formulation– the immersion of the glass (powder and plaque) into SBF and the response of MG-63 cells (resistant osteoblast's cell line) when seeded in plaque. From the powder-test the formation of a precipitate rich in Ca and P was detected, and the pH has stabilized below 7.5 (day 14). Relatively to plaque, the degradation was lower and a layer rich in Ca and P (Brushite-$CaHPO_4 \bullet H_2O$) was also detected. MG-63 cells showed a strong adhesion and proliferation in glass surface (better results to lower roughness), a high growth-cell rate and absence of toxicity (Silva et al., 2008).

The appearance of Ca and P as a precipitate or layer show evidence of mineralization and at the same time biocompatibility, due to the positive responses of medium cell MG-63. Thus, the glass formulation with Ti, Ca and P is a good candidate to biomedical applications, namely bone regeneration.

However, beyond direct contact of the biomaterial with cells, the ions released from the biomaterial also affect bone cells response, but the mechanisms are still unclear (Silva et al., 2008). Thus, it is important to assess this effect in order to complete the information about MG-63 cells behavior with exposition to dissolution products of this novel bioglass.

3. MAIN AIMS

The main objective of the present study is to analyze the effect of the products of dissolution of powder glass (extract) in MG-63 and in Zebrafish (*Danio rerio*) early-life stages, to evaluate the toxicity of ions released from degradation of biomaterial in two models.

In *in vitro* model it was analyzed the viability and proliferation of MG-63 cells over the time (1 day, 3, 7 and 10 days of exposure) after its culture with different dilutions of PBS (*Phosphate Buffered Saline*) extract (0.1%, 0.5%, 1%, 10%, 20% and 50%). Control's concentrations were the same tested for extract, but it was also tested 0% of PBS (Positive Control). After seven days of direct exposition with PBS extract, it was studied the expression of important bone formation genes (GADPH, Col-1, ALP, BMP-2, OPG, M-CSF and RANKL) for selected concentrations (Control: 0% PBS, 20% PBS; and Extract: 0.1%, 10%, 20% and 50%).

In *in vivo* model *it* was detected the activity of different enzymes (LDH, AChE and GST) in Zebrafish larvae exposed to fish water culture extract with six different concentrations (0%, 1%, 2.5%, 5%, 10% and 20%).

For that, the project was developed according the following tasks:

In Vitro Studies

- Dissolution of glass powder in PBS,
- Culture of MG-63 in medium with solutions test and controls,
- Analysis of viability and Proliferation of cells by MTT assay,
- Analysis of morphology of cells by Confocal Laser Scanning Microscopy,
- Detection of the effect of dissolution powder products in expression of genes by RT-PCR,
- Treatment of data.

In Vivo Studies

- Dissolution of glass powders in Zebrafish water sterilized and measure of the pH,
- Eggs exposure during 96 hours and frost at -80ºC until enzymatic assays,
- Analysis of enzymatic activities by enzymatic assays,
- Treatment of data.

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Chapter 2 - *In vitro studies*

1. INTRODUCTION

The cells of bone tissue at all stages of bone formation interact by a functional network. Signals travel around and within a pool of cells, such as proteins (e.g. cytokines), growth factors and transcription factors.

MSCs are bone marrow stromal mesenchymal cells and are the initial of sequential stages. They have the ability to proliferate and to differentiate into different cells (for example adipocytes, osteoblasts, chondroblasts, myoblasts and fibroblasts), according to specific message transmitted by signal, resulting in the expression of morphogenes, the pathways of signals' cascades and the regulation of transcripts (Figure 3 A). The size of cells populations depends on the balance between proliferation, differentiation and apoptosis (Pekkinen, 2008).

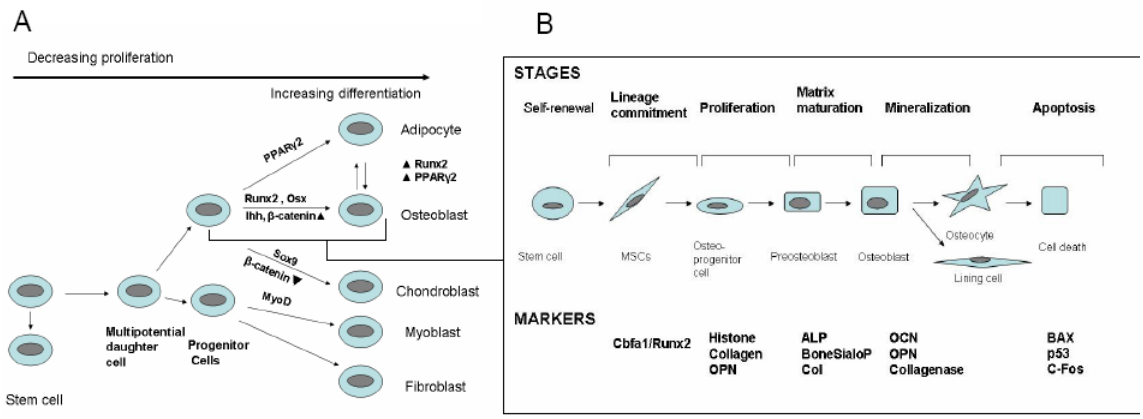

Figure 3: A) Rate of proliferation and differentiation of mesenchymal stem cells by signal molecules. B) Stages of osteoblastogenesis and respective markers (Pekkinen (2008) adapted from Lian et al., (2003)).

Curiously, other characteristic of cells is the **phenotypic plasticity**. According to the expression of transcription factors, these cells can transdifferentiate into other phenotype, such as adipocytes can be converted into osteoblasts, and vice-versa, and the genes involved are PPARγ2 and Runx 2 (Pekkinen, 2008).

Bone morphogenetic proteins (BMPs) belong to TGF-β superfamily of genes and play roles in the regulation of bone process and in the embryonic development (Cho et al., 2009; Sykaras et al., 2003). They appear immersed into matrix prepared to interact with cells in the microenvironment. Its mobility depends on geometrical parameters of delivery, matrix

composition and possible carrier materials (Sykaras et al., 2003). When a fracture healing occurred, the expression of BMP receptors is increased in osteogenic cells near to brittle area (Cho et al., 2009).

The differentiation of osteoblasts occurs into three stages - proliferation, synthesis of extracellular matrix and maturation, and finally mineralization - and it can be distinguished by the expression of different markers. For instance, ALP (alkaline phosphatase), BSP (bone sialoprotein) and Col-1 (collagen type-1) are expressed at initial stage of osteoblasts' differentiation, and PTHR (PTH/PTHrp receptor) and OCN (osteocalcin) are expressed at late stage and at the same time of mineralization (Figure 3 B) (Pekkinen, 2008).

Osteoclasts are formed from fusion of their mononuclear precursors, which are found in bone marrow and peripheral blood. Then they differentiate and play a role in bone resorption (Suda et al. (1996) in Costa-Rodrigues et al. (2010)). There are two important growth factors for *in vitro* osteoclastogenesis: monocyte-colony stimulation factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL) (Boyle et al. (2003) in Costa-Rodrigues et al. (2010)).

The M-CSF is a homodimeric glycoprotein produced by different cells and plays a role in formation of osteoclasts, and also in stimulation of survival, chemotactic response of isolated ones, and in maturation of these cells (Srivastava et al. (1998) and Ross (2006)).

Secondly, in the middle of interaction appears RANK which is a receptor present in membrane of osteoclast precursor (Sun et al., 2009). However, the osteoblasts have the central role, because they synthesize and secrete either RANKL or OPG (osteoprotegerin). There is a competition between these signals (produced at different stages of cells maturity), according to bone tissue requisites, osteoblasts proliferation or resorption, respectively (Figure 4) (Pekkinen, 2008).

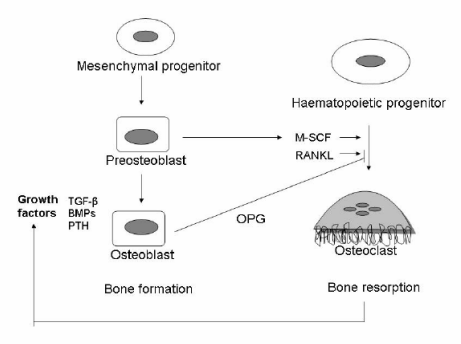

Figure 4: Remodelling of bone and the interactions of cells (Pekkinen, 2008).

More concentration of OPG allowed osteoblasts to prevent osteoclastogenesis, increasing the apoptosis of mature osteoclasts, inhibiting the activation of existing osteoclasts and decreasing its number. After RANK-RANKL connection fulfilled several pathways may development, and subsequently occurs the activation of signals cascades. For instance, it is activated the nuclear factor-kβ and secondly the c-jun N-terminal of protein kinase and subsequently steps culminate in digestion of tissue by osteoclasts (Sun et al., 2009). The osteoclasts attach to bone surface by podosomes and seal the bone matrix in specific circular areas (rugously areas) with border membranes. Next, these cells secrete HCl, to dissolve the mineral content, and lytic enzymes (e.g. cathepsin K), to digest the organic matrix (Boyce et al. (2008) and Vaananen et al. (2008) in Costa-Rodrigues et al. (2010)).

After putting the glass system locally on the bone damaged, the biomaterial will dissolve and some ions will interact with the pool of cells present in the environment. Thus, it is very important evaluate the cells' behaviour exposed to the dissolution products of the glass tested with a great superficial area, to increase the reactivity and the amount of ions released. Some studies supported the ions interaction with cells.

Foppiano et al. (2007) studied the behavior of MC3T3-E1.4 mouse pre-osteoblastic cells exposed to the FGC (Functionally Graded Coatings) of bioactive glass dissolution products in parallel with TCP and uncoated Ti_6Al_4V (titanium alloy where the coating is placed) surfaces. They detected that these products induced an **increase** in the expression of Runx-2 (which is one regulator of early stages of osteoblast differentiation) and a **decrease** in the gene of collagen type I (regulator of osteoblast mineralization) expression (less 20% than on TCPS (Tissue Culture Polystyrene) extract).

Sun et al. (2009) showed that the ionic products of Ca_2SiO_4 coatings into DMEM (Dulbecco's Modified Eagle's Medium) promoted the proliferation of MG-63 cells (it was more significant than control). They also analyzed the cell cycle and concluded that after exposure to this extract G0/G1 phase decreased and S phase increased. Furthermore, the differentiation of MG-63 cells at initial phase was promoted. Relate to osteoblasts-related genes, extract from Ca_2SiO_4 coatings enhanced its expression, and inhibited osteoclastogenesis by the up-regulated OPG and down regulated RANKL.

Neel et al. (2008) tested some properties of maximum content of TiO_2 dopped phosphate glasses (such as physical, surface and short-term response of MG-63) and concluded that it was possible incorporate 15 mol.% TiO_2 into the ternary formulations, preserving the amorphous structure. The degradation rate was significantly reduced, decreasing also the ions released and MG-63 performance was maintained.

This chapter present some testes done in MG-63 cells, an osteoblast-type cell line, resistant, derived from a human osteosarcoma that express a number of features characteristic of osteoblasts.

2. MATERIALS AND METHODS

2.1. PREPARATION OF THE POWDER

$14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ frit was crushed and the powder was sieved to less than 20 µm particle size within specified conditions (compartment with air influx and mask). Some powder was analyzed to check its medium size (granulometry evaluation).

2.2 .PREPARATION OF THE EXTRACT: DISSOLUTION AND CHEMICAL CHARACTERIZATION (MEASURE OF PH AND IONS CONCENTRATION)

Flasks were sterilized at UV radiation and 250 mg of powder were immersed into 10 mL of PBS (0.01M *Phosphate Buffered Saline*: 0.138M NaCl, 0.0027M KCl, pH 7.4 at 25ºC) (Sigma) solution at 37ºC during different times (2 hours, 7 hours, 1 day, 7 days and 14 days). Each solvent was filtrated with 0.25 µm filter and the final pH was measured. The powder was dried at room temperature and stored for further analyses. The extract used in each experimental procedure was that resulting from a 7-day incubation period. Specimen ion leaching to PBS was studied by Inductively Coupled Plasma (ICP) spectroscopy, and the sample was stored at 4ºC. Each flask with extract was sterilized under UV radiation (to use in the biological experiments).

2.3. PREPARATION OF CULTURE MEDIUM AND EXPOSURE TO EXTRACT

MG-63 cells were subcultured in α-MEM (α-Minimum Essential Medium) (Gibco, Invitrogen) supplemented with 10% of FBS (Foetal Bovine Serum) (Gibco, Invitrogen), 50 µg/mL of Ascorbic Acid (Fluka), 2.5 µg/mL of Fungizone (Gibco, Invitrogen) and 100IU/mL of Penicillin, 100 µg/mL Streptomycin) (Gibco, Invitrogen), into a petri dish, in a humidified atmosphere with 5% CO_2 and were incubated at 37ºC overnight.

After, the cells were washed with PBS (Phosphate Buffer Solution) (Sigma) and were detached from the surface with a solution of 0.04% trypsin in 0.25% EDTA solution. The concentration of the resulting cell suspension was measured in Cell's Counter (Celltac, from Nihon Kohden).

Cells were cultured into 96-wells plates at $2x10^4$ cel/cm^2 initial concentration in α-MEM (Gibco, Invitrogen) supplemented as referred above in a humidified atmosphere with 5% CO_2, and were incubated at 37ºC overnight.

The culture medium was removed and replaced by culture medium with different concentrations of extract and PBS (0.1%, 0.5%, 1%, 2.5%, 5%, 10%, 20%, and 0% PBS just for control), in a humidified atmosphere with 5% CO_2; 8 replicates were performed for each experimental situation periods of exposure were 1, 3, 7 and 10 days.

At the end of each exposure period, cultures were evaluated for viability/proliferation (MTT assay) and were observed by Confocal Laser Scanning Microscopy (CLSM).

2.4. MTT ASSAY

Formazan crystals are the product of reaction between the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) react and the tetrazolium ring, produced by mitochondrial dehydrogenases of living cells. So, its amount is proportional to the number of viable cells present (Sun et al., 2009).

0.5 mg/mL MTT was added to each well and incubated during 3 hours at 37ºC. The medium was removed and 100 µL of DMSO were added. The absorbance' values were measured at 550 nm on an ELISA reader (SynergyHT BioTeK).

2.5. CONFOCAL LASER SCANNING MICROSCOPY

First, the supernatant was removed and cells were washed twice with PBS and fixed with 3.7% of Formaldeide during 15 minutes at room temperature. Next, cells were washed twice with PBS and stored at 4ºC until labelling.

For labeling cells were permeabilized with 0.1% Triton X-100 solution in PBS during 5 minutes.

The supernatant was removed and 10 mg/mL BSA (albumin solution) with 1 µg/mL of RNAse in PBS was added, and incubated during 1 hour. The solution was removed and phalloidin solution (5 U/mL, AlexaFluor® 488 (Invitrogen)) was added to stain F-actine filaments, incubating for 20 min in the dark at room temperature. The supernatant was removed and Propidium Iodide solution (10 µL/mL in PBS (Sigma)) was added to stain nucleus. The period of incubation was 10 min, in the dark and at room temperature. After, the supernatants were removed and finally cells were covered with mounting

medium (20mM Tris pH 6.0, 0.5% N-propyl gallate, 90% glycerol) to preserve fluorescence' signal. CLSM was performed in Leica SP2 AOBSSE a (Leica Microsystems, Germany) Laser Scanning Confocal Microscope.

2.6. REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR):

RT-PCR was used to amplify the complementary DNAs converted from mRNA encoding different human gene products by the enzyme Transcriptase Reverse.

First, Total RNA was extracted from 7th day cultures and selected concentrations (Control 0% PBS, Control 20% PBS, 0.1 % Extract, 1 % Extract, 10 % Extract, 20 % Extract) using the Nucleospin® RNA II kit (from MACHEREY-NAGEL) and 2 µL was quantified in ELISA equipment (Synergy HT, from BioTek with Take3 module).

RT-PCR was performed using TITAN ONE TUBE RT-PCR SYSTEM kit (Roche). Optimal concentration of each reagent (water RNAse free, Buffer, dNTP, DTT, primers Forward and Reverse, RNA and Enzyme) was used according to the manufacturer's instruction with a total volume of 25 µL, and the reactions were made for 7 specific primers, namely glyceraldehydes-3-phosphate dehydrogenase (GAPDH), type I collagen (COL-1), ALP, BMP-2 (Sun et al., 2009), OPG, RANKL (Sun et al., 2009), and M-CSF.

The Thermocycler (Tpersonal, from Biometra) was programmed with the adequate temperature, number of cycles and duration of cycles for each reaction (Table 1).

Table 1: RT-PCRs' Programs (for osteoblasts)

	Phase	Temperature (ºC)	Duration of cycles	Cycles	Primers
1		50	0h30m		
2		94	0h2m		
3		94	0h0m30s		
4	Annealing	50	0h0m30s		M-CSF
		55	0h0m30s		GADPH,Col-1,ALP, RANKL
		60	0h0m30s		BMP-2, OPG
5	Elongation	68	0h0m45s	3 | 4	
6		94	0h0m30s		
7	Annealing	50	0h0m30s		M-CSF
		55	0h0m30s		GADPH,Col-1,ALP, RANKL
		60	0h0m30s		BMP-2, OPG
8		68	0h0m45s	6 | 29	
9		68	0h7m		
10		4	stop		

The electrophoresis was performed with 7.5 µL of samples amplified with 1.5 µL of loading buffer and 6 µL of weigh molecular marker (MW 4) that were pipetted in wells of an electrophoresis 1% agarose gel and it was run through 1x TAE (running buffer) at 125 V during 40 minutes.

After this, gel was photographed in the UV transiluminator and band intensity was measured with the help of a computer program MacBiophotonics ImageJ.

The data media was normalized against GADPH and gene expression was compared between treatments, and was represented by a bar chart graph.

2.7. STATISTICAL ANALYSIS

A nested ANOVA test was used to analyze differences associated with two endpoints: cells' absorbance values (ABS) derived from MTT assay and Cell's Growth Rate (CGR) and verified the differences between groups. The hierarchical model used Concentration and Extract (presence of absence) as fixed factors while Timeframe was used as the nested factor. A level of significance of 0.05 was used for all statistical tests.

3. RESULTS AND DISCUSSION

3.1. pH ANALYSIS

The pH values decreased from 7.1 to 6.4 and stabilized on last days of incubation (168 hours and 336 hours) (Figure 5).

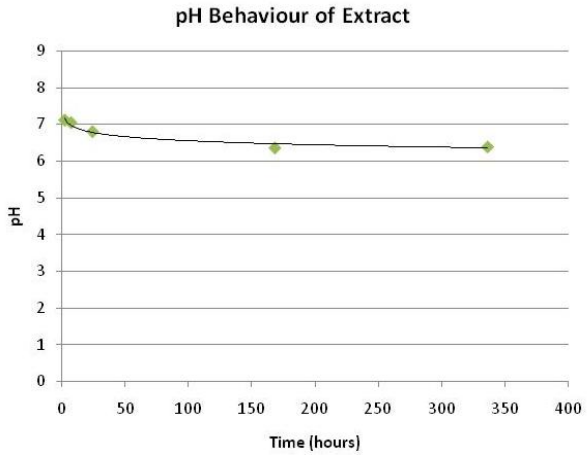

Figure 5: pH values over dissolution time in PBS Extract.

This behaviour was similar to results reported by Silva et al. (2008) regarding to the dissolution of powder in SBF (Simulated Body Fluid). The pH stabilized below 7.5 (day 14).

3.2. ICP ANALYSIS

Results of ICP analyzes are showed in Figure 6. Along the time, releasing of Ti and Ca ions increased. However, no one pattern was detected for P, the concentration range between 200 and 300 mg/L.

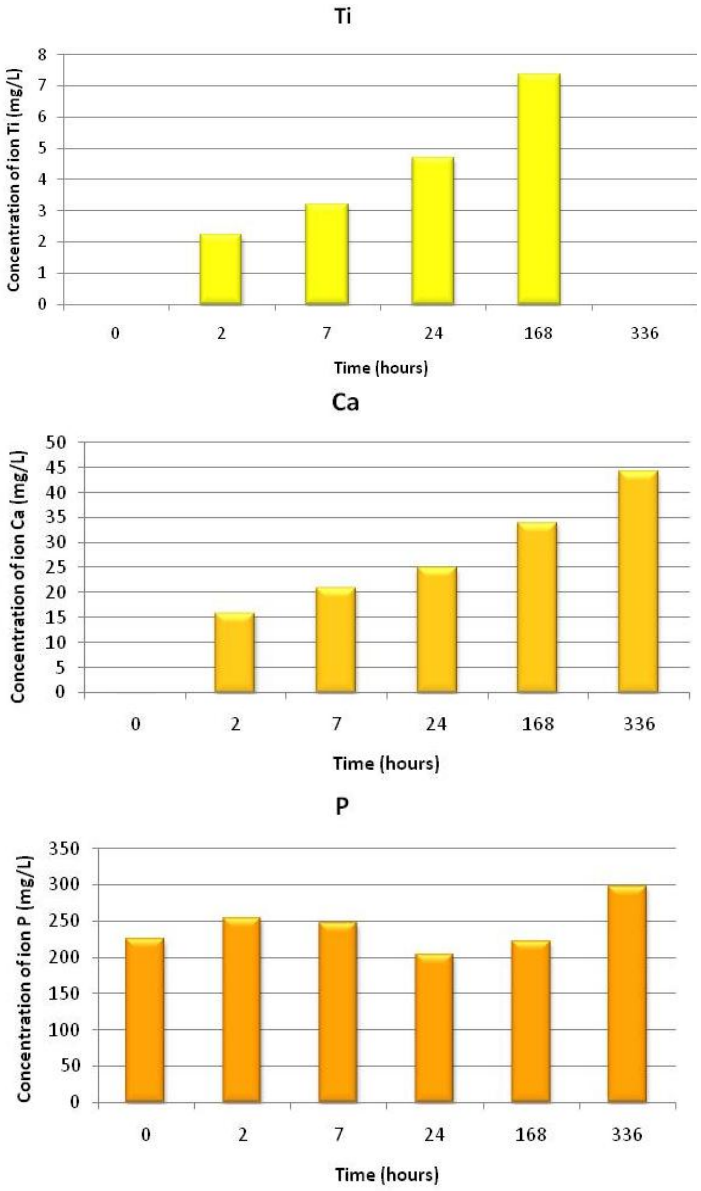

Figure 6: Concentration of principal ions products from dissolution of powder glass in PBS measured by ICP.

The release of P and Ca is consistent with the formation of a precipitate rich in Ca and P when powder-glass was dissolved in SBF (Silva et al., 2008). ICP measurements detected other ions, such as K, Na, Ba and B (data not shown in the present study). The extract selected for further tests is the 7^{th} day solution.

3.3. CONFOCAL LASER SCANNING MICROSCOPY

Cell cultures exposed to $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ powder extracts and the respective PBS controls were stained for F-actin cytoskeleton and nucleus and were observed by CLSM. Results regarding the cultures exposed to the extracts at 0.1%, 5% and 50% are presented in Figure 7.

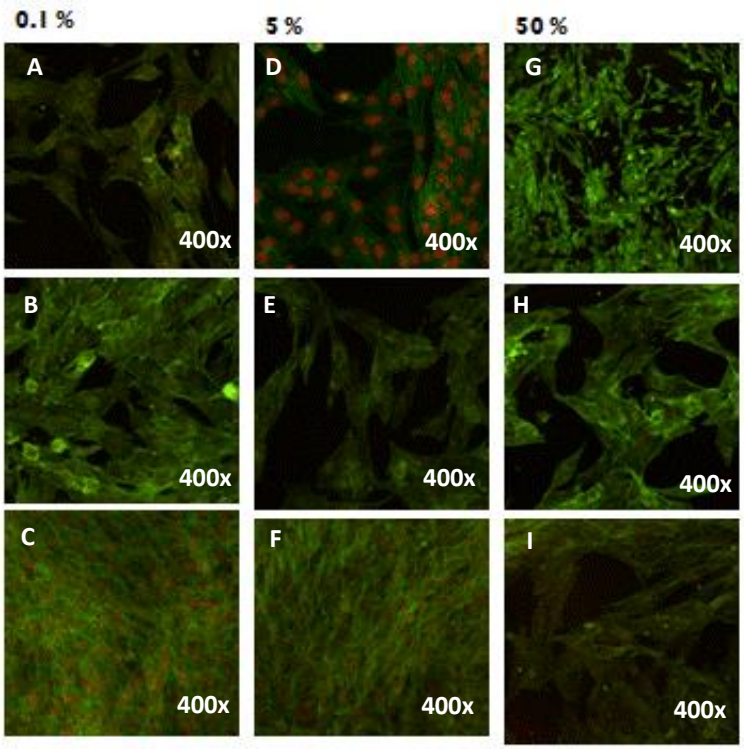

Figure 7: Legend: A) 0.1% PBS, day 3; B) 0.1% Extract, day 3; C) 0.1% Extract, day 7; D) 5% PBS, day 3; E) 5% Extract, day 3; F) 5% Extract, day 7; G) 50% PBS, day 3; H) 50% Extract, day 3; I) 50% Extract, day 7.

Cultures performed in the presence of the extracts presented a morphology and a F-actin cytoskeleton organization similar to that observed in the cultures maintained with the same concentrations of PBS (control). Also, in both situations, cells maintained extensive cell-to-cell contact and presented a similar pattern of cell growth. In addition, the number of cells increased with the culture time. However, cultures exposed to the extract at 50% showed a lower number of cells compared to the respective PBS control.

3.4. MTT ASSAY

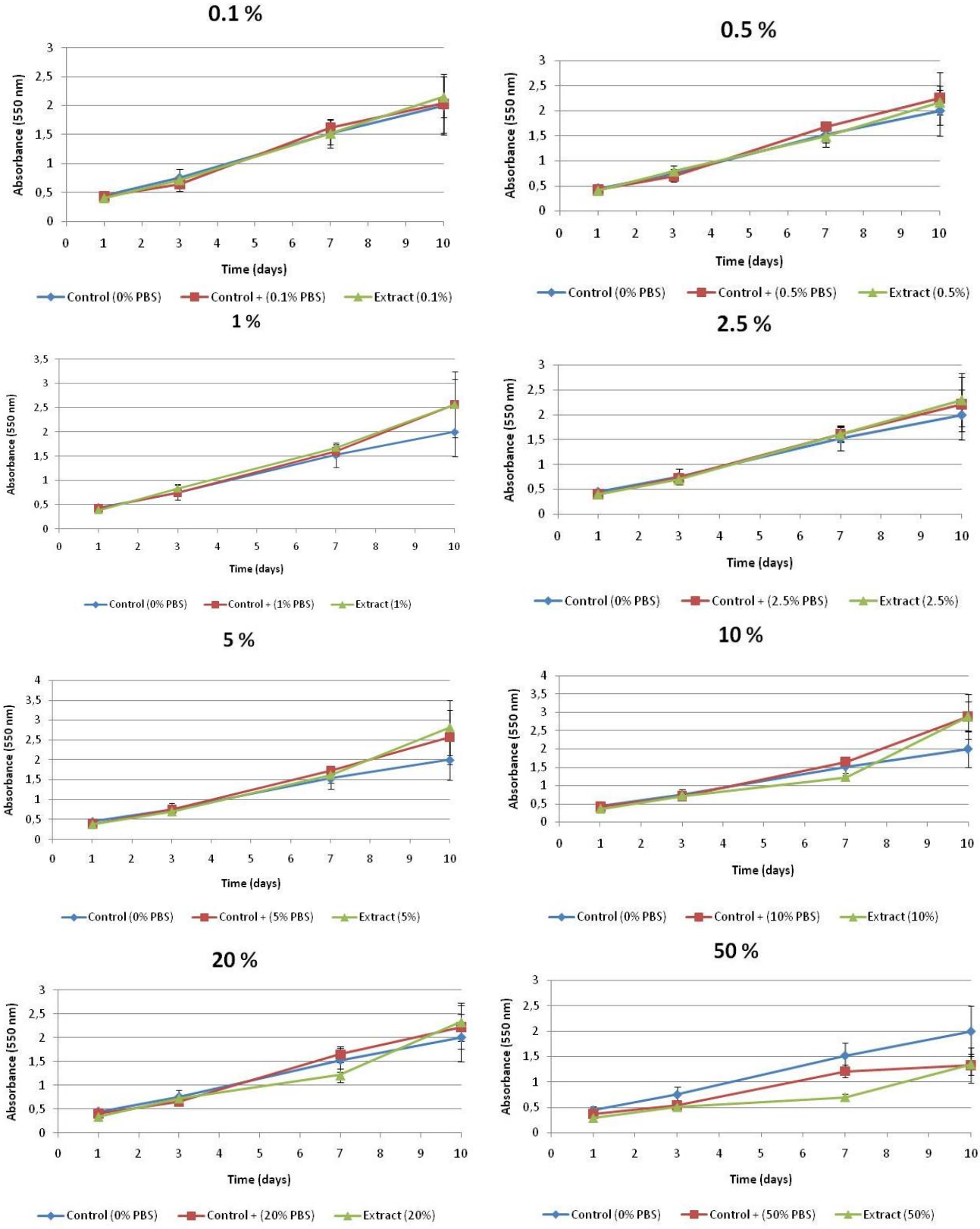

Figure 8: Cell viability and proliferation (MTT assay) of MG-63 cell cultures exposed to $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ powder extracts in PBS (0.1 - 50%) and PBS (control) (0 - 50%).

MG-63 cell cultures were cultured for 10 days and were exposed to a concentration range, of $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ powder extracts (0.1 - 50%), prepared in PBS. MTT assay was used to assess cell viability/proliferation. Cultures performed in the presence of similar concentrations of PBS were used as control. Results are shown in Figure 8.

Exposure of MG-63 cells to the extract at concentrations up to 5% did not influence cell viability/proliferation, as the MTT results were similar to those observed in the cultures performed in the presence of the same concentrations of PBS. However, exposure to 10 – 50% of the extract resulted in a concentration-dependent inhibition of cell viability/proliferation in the first days, as, at day 7, the MTT values were lower than those observed in the presence of the same concentrations of PBS. This inhibitory effect was more pronounced between days 3 and 7. However, cells were able to recover from this negative effect and, at day 10, the MTT values were similar to the PBS control. Thus, compared to control, cell growth rate in the cultures exposed to the extracts was lower between days 3 and 7 and higher between days 7 and 10.

Cell viability

Hierarchical comparisons of cell viability using a nested ANOVA showed statistically significant differences between concentrations [F(7, 355)=37.43, *P*=0.000] and between the PBS and PBS plus extract (subgroup: Extract [F(1, 355)=10.43, *P*=0.001] and Concentration*Extract [F(7, 355)=2.11, *P*=0.042]).

The concentrations 50% and 20% of extract were statistical different, and 0.1%, 0.5%, 2.5% and 10% weren't statistical different from 20% of extract. 1% and 5% of extract were also statistical different.

Timeframes 1 (until day 1), 2 (from 1 to 3 days), 3 (from 3 to 7 days) and 4 (from 7 to 10 days) were statistical different.

Tukey simultaneous tests demonstrated that the viability of MG-63's cells for extract was statistically different from control (PBS), and subsequently values comparison showed that was lesser than control (*p*=0.0007).

The concentration of extract caused a different effect in MG-63 cells, when they were exposed to 50% and 5%, and the number of viable cells was lesser than control. The time of exposure was also interfered in this parameter.

Cell Growth Rate analysis

The variability of medium values between concentrations and between timeframe were statistical significant ([F(7, 270)=2.46, *P*=0.019, and F(2, 270)=47.93, *P*=0.000], respectively). Statistically significant differences were found between concentrations (Figure 9).

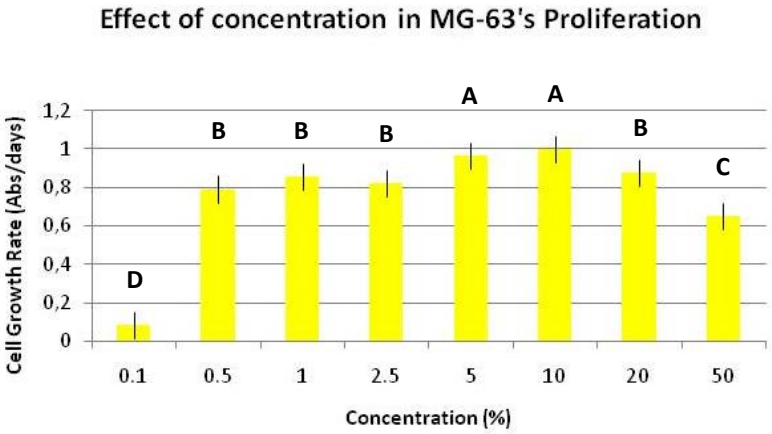

Figure 9: Effect of concentration in MG-63's proliferation (letters denote groups identified by the post hoc Tukey test).

The timeframes 1 (from 1 to 3 days), 2 (from 3 to 7 days) and 3 (from 7 to 10 days) were statistical different (Figure 10).

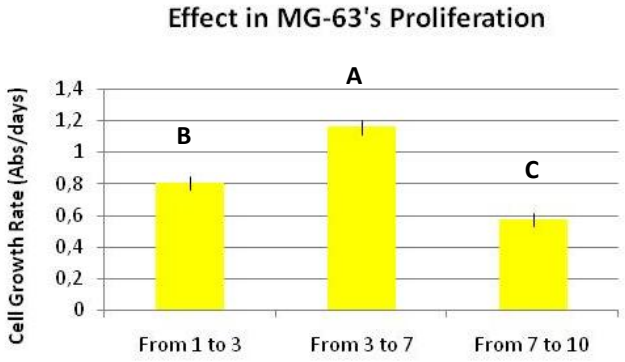

Figure 10: Effect of time in MG-63's proliferation (letters denote groups identified by the post hoc Tukey test).

Thus, with this analyzes it can conclude that the extract with 50% promoted a different effect in MG-63's proliferation in comparison with 5% or 10%, and along the time the proliferation of cells was different.

3.5. RT-PCR

RT-PCRs were done to evaluate the expression of important genes implicated in bone formation and remodeling, namely Collagen type 1, ALP, BMP-2, OPG, M-CSF and RANKL. Results are shown in Figure 11. The intensity of bands was measured and was normalized against GADPH (control). The transcriptional level of genes is shown in Figure 12.

Cultures expressed low levels of RANKL, and the quantification of this gene was not possible.

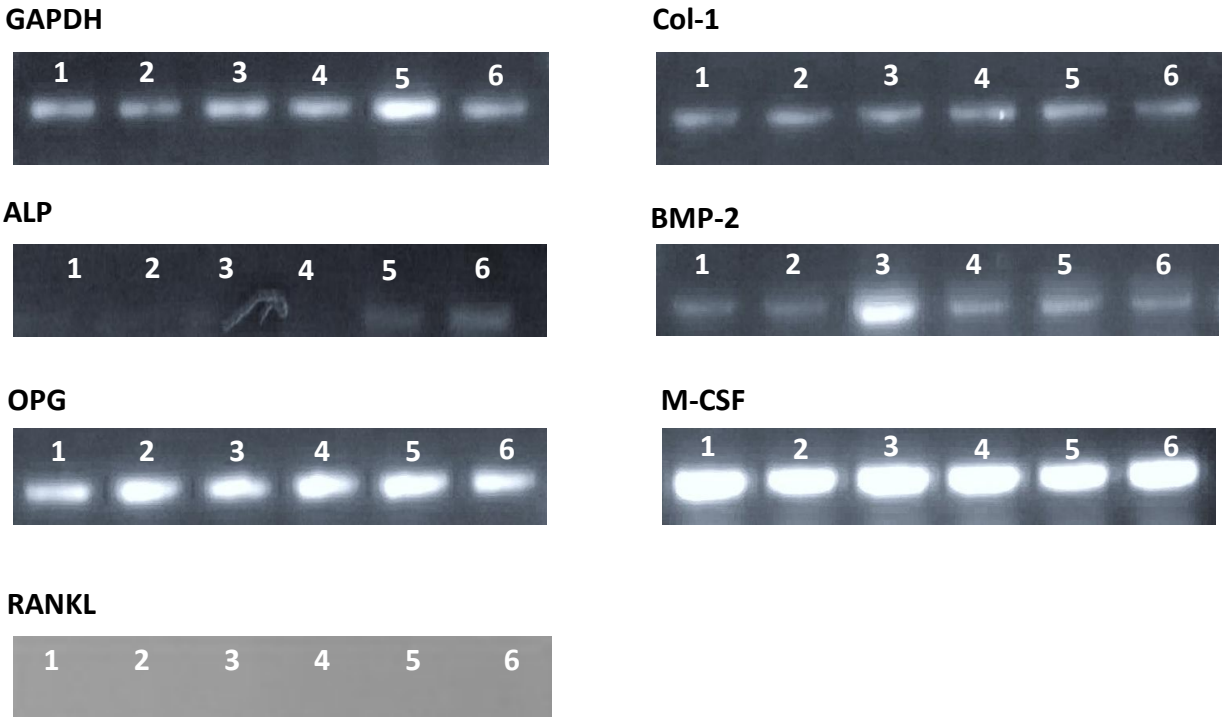

Figure 11: Expression levels of mRNAs of genes implicated in bone formation and remodelling, such as GADPH (control), Col-1, ALP, BMP-2, OPG, M-CSF and RANKL of MG-63 cells cultures exposed to $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ powder extract in PBS and PBS (control). Legend: 1- Control (0% PBS), 2- Control (20% PBS), 3- 0.1% Extract, 4-1% Extract, 5-10% Extract, 6-20% Extract.

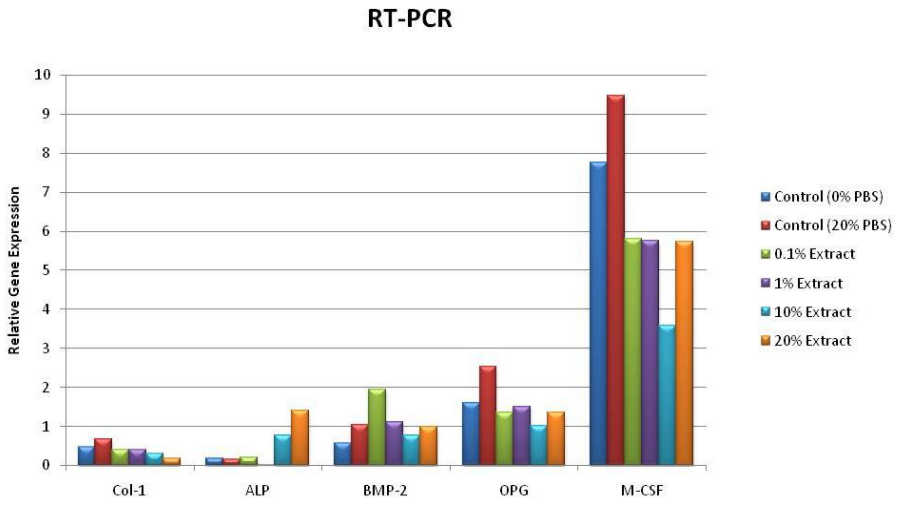

Figure 12: Relative gene expression of Col-1, ALP, BMP-2, OPG and M-CSF in MG-63 cells cultures exposed to $14TiO_2•43P_2O_5•43CaO$ powder extract in PBS and PBS (control), using RT-PCR.

ALP's expression had a dose-response behavior, and it was up-regulated and higher for 10% and 20% of Extract. ALP is an early phenotype marker of osteoblasts differentiation. Also, stimulation of BMP-2 appeared evident in the cultures exposed to 0.1% and 1% of extract. BMP-2 is known to stimulate the proliferation and differentiation of mesenchymal and osteoprogenitor cells, and are angiogenic (Cho et al., 2009). Expression of Col-1, OPG and M-CSF was down-regulated (especially M-CSF) and no significant differences were found regarding the tested extract concentrations.

The results showed that the ionic products from the dissolution of $14TiO_2•43P_2O_5•43CaO$ powder glass cause a slight dose-dependent inhibitory effect in the cell viability/proliferation of MG-63 osteoblast-like cells within the concentration range 10% to 50%. The negative effect was evident between days 3 and 7, suggesting a time-dependent cumulative deleterious effect in the cell growth. However, cells were able to recover from this initial negative effect, as reflected by the significant increase in the cell growth rate between days 7 and 10. Observation of the cultures by CLSM showed that the extract did not affect the cell morphology and the organization of the F-actin cytoskeleton within the tested concentration range. RT-PCR analysis suggested that the extract, at selected concentrations, stimulated the expression of genes related to bone formation (ALP and BMP-2) and down-regulated the expression of the gene M-CSF which is related to the osteoblast-mediated osteoclastogenesis, suggesting a net positive effect in bone formation events.

The results suggested that the tested powder is promissory, but more studies are needed, due to the complexity of cells network, with more realistic models.

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Chapter 3 - *In vivo studies*

1. INTRODUCTION

1.1. THE IMPORTANCE OF ZEBRAFISH IN BIOMINERALIZATIONS' STUDIES

Zebrafish is a small freshwater cyprinid (Oliveira et al., 2009) from the rivers of Northern India, and is currently used as model organism. Today, there are a research community of more than 500 laboratories and 4,000 researchers registered on the Zebrafish Information Network (ZFIN), which is an international web-resource (Cooke et al., 2007). Short embryological period and the events are similar between all vertebrates, not just in molecular genetics and toxicology, but also in studies of developmental biology and physiology (Oliveira et al., 2009). Each female can produce hundreds of eggs for clutch and are easily manipulated and fertilized (Gerhard, 2003). The embryos are small, transparent and their development is rapid and externally. Cells, tissues and organs can be monitored in real time and *in vivo* with the help of a stereomicroscope. So, they are indicated for phenotypic analysis during embryogenesis and organogenesis. Compounds tested can be exposed directly to the embryos, dissolved into the medium (Fleming, 2007), and multiple targets can be studied simultaneously within a pathway (Cooke et al., 2007). Furthermore, individual embryos are permeable to several small molecules (Gerhard, 2003). The screen of compounds for osteoporosis therapy in zebrafish can replace the rodent models, because the lesser duration of test - 1 week, using intact animals, in comparison with long period in rodents, divided in two moments, the induction of bone loss and the treatment actuation (the maximum is 10 weeks and up 1 year, respectively) (Fleming, 2007). For instance, the exposure of embryo to ethanol, during its development, can result in craniofacial abnormalities, optic nerve damaged and later learning. It is advantageous the study of drugs effects using Zebrafish model, specially with developing larvae due to high regenerative capacities (not present in humans) (Cooke et al., 2007).

The development of bones is like in human and during the adult phase the skeleton is constantly change. Actually, scientists have been studied the biomineralization (formation of inorganic minerals by living organisms) using Zebrafish individuals. The skeleton has crucial roles which concern to attach the muscles and movement and represent a source of calcium ions for muscle contraction and nerve impulse transportations (Neues et al., 2006).

Neues et al. (2006) studied the replacement permanent of pharyngeal dentition and concluded that the formation and resorption is very quick (9-11 days). This dynamic process is controlled by environmental factors such as microgravity and radiation, and can be monitored by micromorphological and microanalytical methodologies, namely Synchrotron radiation microcomputer tomography (SRμCT), scanning electron microscopy, polarized light microscopy, and energy dispersive X-ray analysis.

1.2. BIOMARKER'S ACTION

Studies in Zebrafish revealed that the activities of Acetylcholinesterase (AChE) is important in neuronal and muscular development (Behra et al., 2002; Hanneman et al., 1992), of Glutathione S-transferase (GST) appear in the biotransformation of several pollutants (Otitoju et al., 2006; Monteiro et al., 2006; Oost et al., 2003), and of Lactate Dehydrogenase (LDH) in conditions of chemical stress (when the supply of high levels of energy is needed) (Oliveira et al., 2009). According to Oliveira et al. (2009), the early-life stages revealed much more information than adult fish test of triclosan (a biocide used in personal care, acrylic, plastic and textile products).

2. MATERIALS AND METHODS

2.1. PREPARATION OF THE POWDER

A glass with the composition (14.48 mol% TiO_2, 42.76 mol% P_2O_5 and 42.76 mol% CaO) $14TiO_2 \bullet 43P_2O_5 \bullet 43CaO$ was prepared as described elsewhere (Silva et al., 2008), poured as a frit and crushed. The powder was sieved to less than 20 µm particle size within specified conditions (compartment with air influx and mask) and the medium size checked by granulometric evaluation.

2.2. PREPARATION OF THE EXTRACT: DISSOLUTION AND CHEMICAL CHARACTERIZATION (MEASURE OF PH AND IONS CONCENTRATION)

Flasks were sterilized at UV radiation and 250 mg of powder were immersed into 10 mL of solution (Water from fish-system sterilized in autoclave) at 37ºC during different times (2 hours, 7 hours, 1 day, 7 days and 14 days). Each solvent was filtrated with 0.25 µm filter and measured the final pH. The powder removed was dried at room temperature and stored for further analyses. Specimen ion leaching to Zebrafish Water was studied by Inductively Coupled Plasma (ICP) spectroscopy, and was stored at 4ºC. The extract used in each experimental procedure was the 7^{th} day. The pH of extract was righted to range 7.4 – 7.5 with NaOH and shaking overnight. After 4 hours, 100 µL was added to solution and mixed, shaking for 30 minutes. Each flask with extract was sterilized under UV radiation (already to use).

2.3. TEST ORGANISM

Zebrafish's (*Danio rerio*) culture are maintained in carbon-filtered water complemented with salt "Instant Ocean Synthetic Sea Salt", at 27.0 ± 1ºC and under a 16:8 h light: dark photoperiod cycle (Conductivity: 550 ± 50 µS, pH 7.5 ± 0.5 and dissolved oxygen > 95% saturation) at the Department of Biology, University of Aveiro (Portugal). Adult fish (at mate age) are fed twice daily with commercially available artificial diet (ZM 400 Granular). There is a facility to obtain Zebrafish's eggs (used in this study) (Domingues et al., 2010). The following ions are present in the zebrafish culture water: Ca, P, K, Mg, Na, Sb, Si and Zn).

2.4. EXPOSURE IN EARLY LIFE STAGES

The early life stages assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) test (OECD, 2006). Zebrafish's eggs were collected within 30 minutes after natural mating and washed in water from the system and selected at Blastula period under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500).

Embryos were immersed into concentrations of pH adjusted-extract diluted in water from system sterilized in autoclave - 0 % (C0, Control), 1% (C1), 2.5% (C2), 5% (C3), 10% (C4) and 20% (C5). Two experimental procedures were made, with different assembly. Firstly, it was used 10 replicates of 6-well plate, where was pipetted 15 mL of solution test per well and it was put 10 eggs per well. However some evaporation occurred, so the initial concentration wasn't the same (saturated solution) because it was added more solution, otherwise larvae will die. Other procedure was made into 6 vans, and it was selected 250 eggs and pipetted into each recipient with respective solution test (100 mL), covered with parafilm.

Eggs were incubated in an appropriate room at constant temperature (28.0 ± 2ºC). Embryos and larvae were observed daily under the same stereomicroscope and died ones were discarded. The test finished at 96 hours and clusters of 10 larvae were frozen in cryopreservation tubes with 0.6 mL of PBS with pH 7.4 at -80ºC until enzymatic analysis. After exposure solutions tested were analyzed by Inductively Coupled Plasma (ICP) spectroscopy.

2.5. BIOMARKERS DETERMINATION

Samples were defrosted on ice and homogenized (with the homogenizer Ystral GmbH D-7801) and each homogenate was divided into two aliquots, one was stored at -80ºC and the other one was spinning at 4ºC and 6,000 rpm, during 4 minutes, into a refrigerated centrifuge (at 4ºC). The supernatant (enzyme extract) was used for determination of AChE and GST activities.

Firstly one standard curve was made with 1 mg/mL γ-globulin of bovine for the initial protein quantification at 600 nm with an adaptation to microplate of Bradford's method. Samples were diluted in buffer solution to obtain the concentration desired for each assay.

Enzymatic activities were determined in quadruplicate, in a Labsystem Multiskan EX microplate reader (for AChE and GST assays), and expressed as nanomol of substrate hydrolyzed per minute per mg of protein.

AChE activity was made at 414 nm after 15 minutes of reaction, according to the adaptation to microplate of Ellman's method (Ellman et al., 1961, in Guilhermino et al., 1996), using 0.05 mL of homogenate diluted and 0.25 mL of the reaction solution (0.1M Phosphate Buffer (pH 7.2), 0.075M acetylcholine solution (ACH) and 10 mM 5,5'-ditriobis-2-nitrobenzoic acid (DTNB)).

Relate to GST activity, it was determined at 340 nm by the methodology of Habig et al., 1981, adapted to the microplate by Frasco et al., 2002, using 0.1 mL of sample diluted and 0.2 mL of the reaction solution (0.1 M Phosphate Buffer (pH=6.5), 10 mM of 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM of reduced glutathione (GSH)). The reaction was run for 5 minutes (with periods of 20 seconds) with an increase in the absorbance.

On the day of enzymatic assay, the homogenates stored were defrosted and centrifuged at 6000 rpm for 3 minutes into a refrigerated centrifuge (at 4ºC). In the supernatant LDH activity was measured at 339 nm and determined according to the modifications of Diamantino et al., 2001, of Vassault's method (1983), using 0.04 mL of homogenate diluted, 0.250 mL of Tris/NaCl/NADH and 0.04 mL of Tris/NaCl/Piruvate. The reaction was read continuously during 20 seconds until 5 minutes.

2.6. STATISTICAL ANALYSES

One-way ANOVA test (Dunett or Dunn's test) was done to analyze the significance of medium activities of each enzyme and verified the differences between concentrations.

3. RESULTS AND DISCUSSION

3.1. pH ANALYSIS

The behavior of pH value was different when immersed in water from Zebrafish culture, due to different salinity compositions of solutions and other compounds.

pH decreased from 5.5 to approximately 3 and it was quite different from control (range between 7 and 8) (Figure 13).

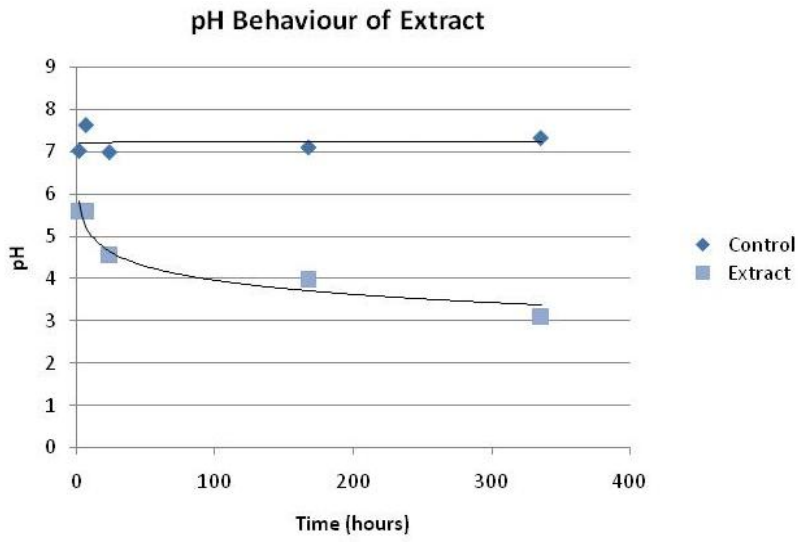

Figure 13: pH values over dissolution times in Zebrafish-water Extract.

Extract solution was adjusted to 7.4-7.5 pH and its dilutions were tested in Zebrafish embryos during its development until larvae.

3.2. ICP ANALYSIS

Figure 14 presents the concentrations of ions Ti, Ca and P dissolved in solvent along the period of incubation. It can detect a common pattern, the amount of ions released increased until 168 hours (7^{th} day) and decreased between 168 hours and 336 hours.

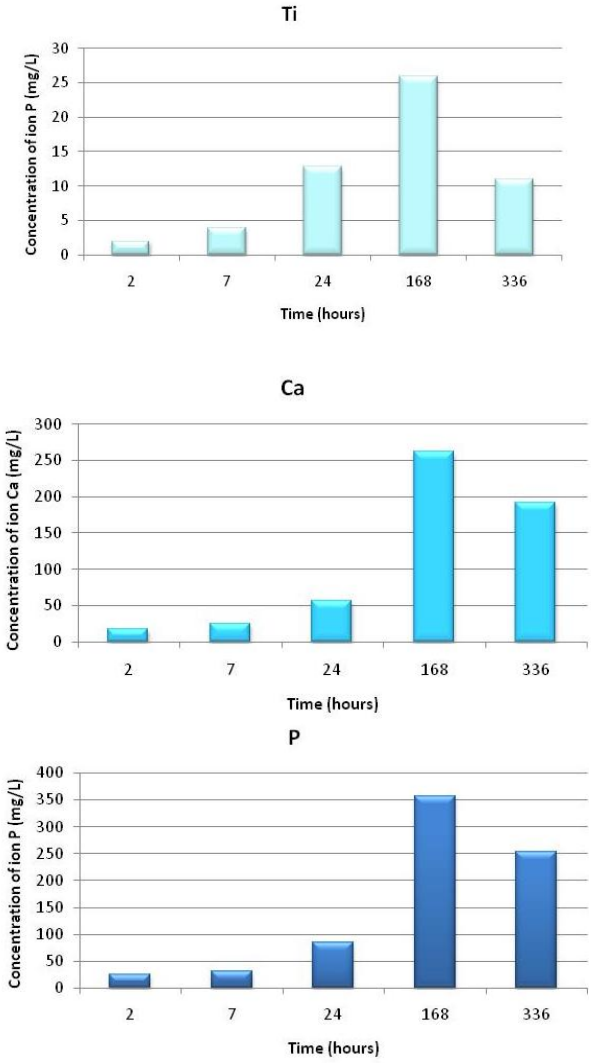

Figure 14: Concentration of principal ions products from dissolution of powder glass in Zebrafish-water by ICP measured.

The extract selected for further tests (7th day) was adequate, according to maximum ion concentrations released to solvent. Probably, between 7 and 14 days of incubation occurred precipitation of dispersed particles or the chemical form of ions differs and ICP detection didn't registered it. For example, the particles of nano-TiO_2 can aggregate into aquatic environment and the size and shape determines the degree of toxics effects. The pH of solution surrounded, the ionic strength and if the ion is inorganic or organic affected the aggregation process (Sharma, 2009). In control (Zebrafish water without powder glass) were registered below detection limit for Ti (BDL= 0.0003 mg/L), 7.57 mg/L of Ca and 0.98 mg/L of P. Values obtained were above that observed in samples incubated with powder glass, confirming the occurrence of ions released from powder glass and its reactivity. ICP

measurements also detected other ions, such as K, Mg, Na and Si (data not shown in the present study).

Figure 15 and Figure 16 show results of ICP analyzes of final solutions, after each toxicity test (1 and 2, respectively).

Between 5 and 20% occurred more expulsion of ions Ca and P from embryo/larvae or them remain into wells, didn't uptake by organism. The upper amounts of Ti are evident for higher concentrations of test solution, suggesting more elimination of this ion at 20% of extract exposure. However, these results aren't predictive, because real concentration doesn't know (solutions test were added to evaporated wells) (Figure 15).

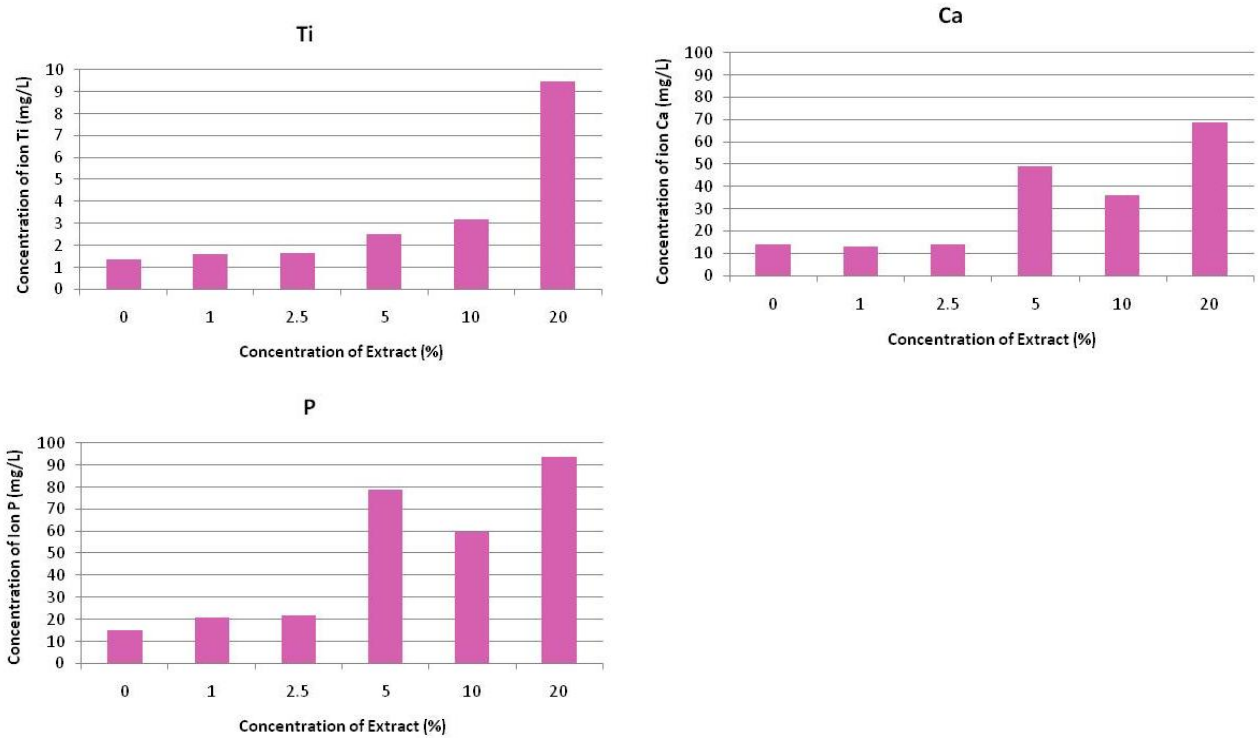

Figure 15: Concentration of ions Ti, Ca and P after embryos/larvae Zebrafish exposure (Test 1), measured by ICP.

In the second test, results suggest higher levels of ions excreted or remain into bath with increase of extract concentration for ions Ti, Ca and P (Figure 16).

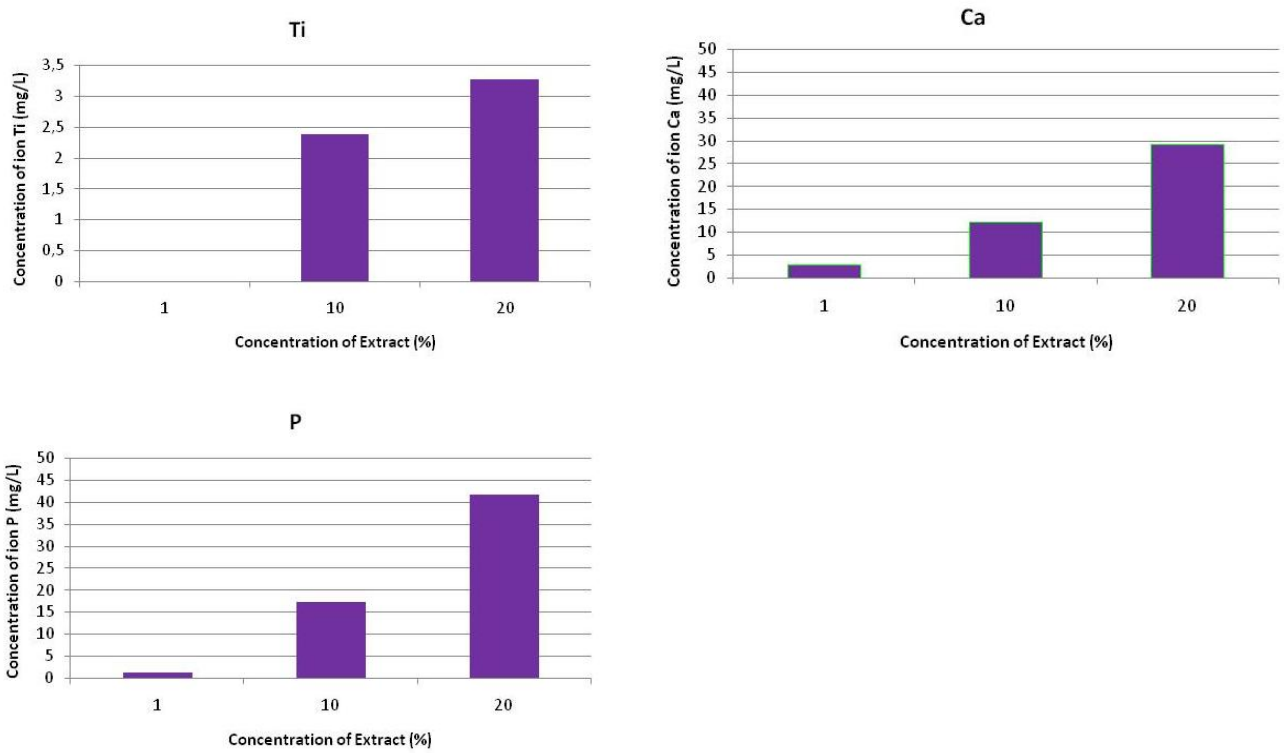

Figure 16: Amounts of ions Ti, Ca and P after embryos/larvae Zebrafish exposure (Test 2), measured by ICP.

The mechanism of action is unknown, probably there is a cambium of ions from exterior solution to embryo inner or larvae.

3.3. BIOMARKERS ASSAYS

Figure 17 and Figure 18 show biomarkers activities in test 1 (saturated solution tested in 6-well plates) and in test 2 (defined concentrations tested in 6 vans), respectively.

Test 1

AChE activity was significantly lesser than control, for eggs exposure to 1%, 2.5%, 5%, 10% and 20% (Dunnett's test, $p<0.001$), demonstrate that occurred an inhibition on activity, suggesting a disruption of the nervous system. However, relate to LDH activity, just for exposure to 2.5% of extract was statistically higher than control (more energy levels was

required to cellular performance due to chemical stress) (Dunnett's Method, *p*=0.007). GST activity was statistically significant for 10% and 20% exposure to extract and it was lesser than control with increased of concentration (H(5)=27.567, *P*=<0.001). The two higher concentrations selected promoted GST activity inhibition and it can damage organism. According to Otitoju et al. (2007) reduction in GST activity may represent an indicator of environmental toxicants. Glutathione is a protective antioxidant, detoxify and eliminate xenobiotics (pesticides) in the beginning of the mercapturic acid pathway (Otitoju et al., 2007).

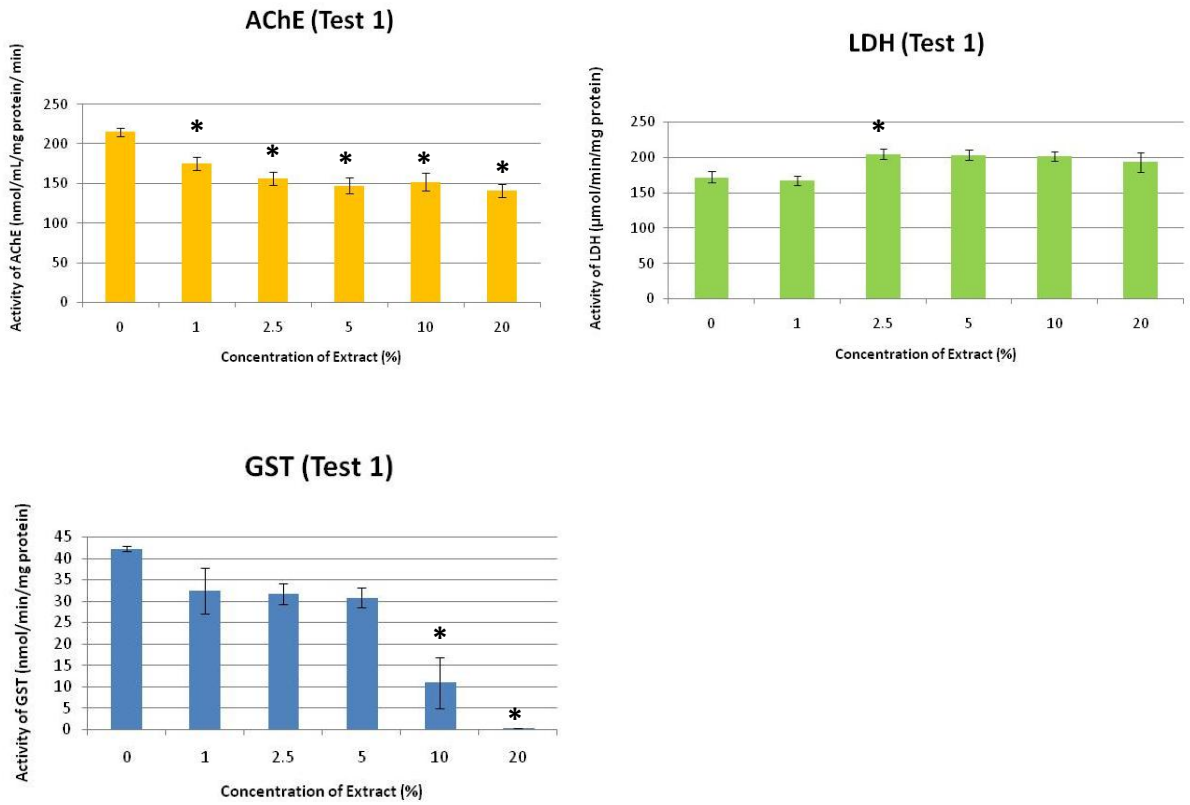

Figure 17: Variation of AChE, LDH and GST activities respectively (mean value ± standard error) on Zebrafish larvae after 96 hours of exposures to water extract, for Test 1. *Asterisks* mean significantly different from control treatment (AChE: Dunnett's test *P*<0.001; LDH: Dunnett's test *q'*=2.685; GST: Dunn's test *P*=<0.001).

Test 2

For AChE activity, the medium values wasn't statistical different between treatments. The tendency of results is controversial, because there isn't data that support a stimulatory effect of AChE between 1% and 5% of Extract. More experimental procedures are need for analyze. For instance, the results showed for test 1, aren't excitatory and the concentration of solutions tests were higher (solutions saturated), besides the experimental designs to be different. LDH activity was significantly lesser than control for 10% and 20% of extract exposures (Kruskal-Wallis Test, *P*=<0.001), suggesting that LDH activity was affected and the detoxification mechanisms weren't well accomplished. Increased levels of activity have been related with chemical stress conditions (Oliveira et al., 2009). Relate to GST, the values medium of activities weren't statistically different between treatments (F(5,51)=0.986, *P*=0.435). So, the concentrations of extract tested didn't affect the GST activity, so its detoxification pathway wasn't activated when Zebrafish embryos and larvae were exposed to selected dilutions of extract.

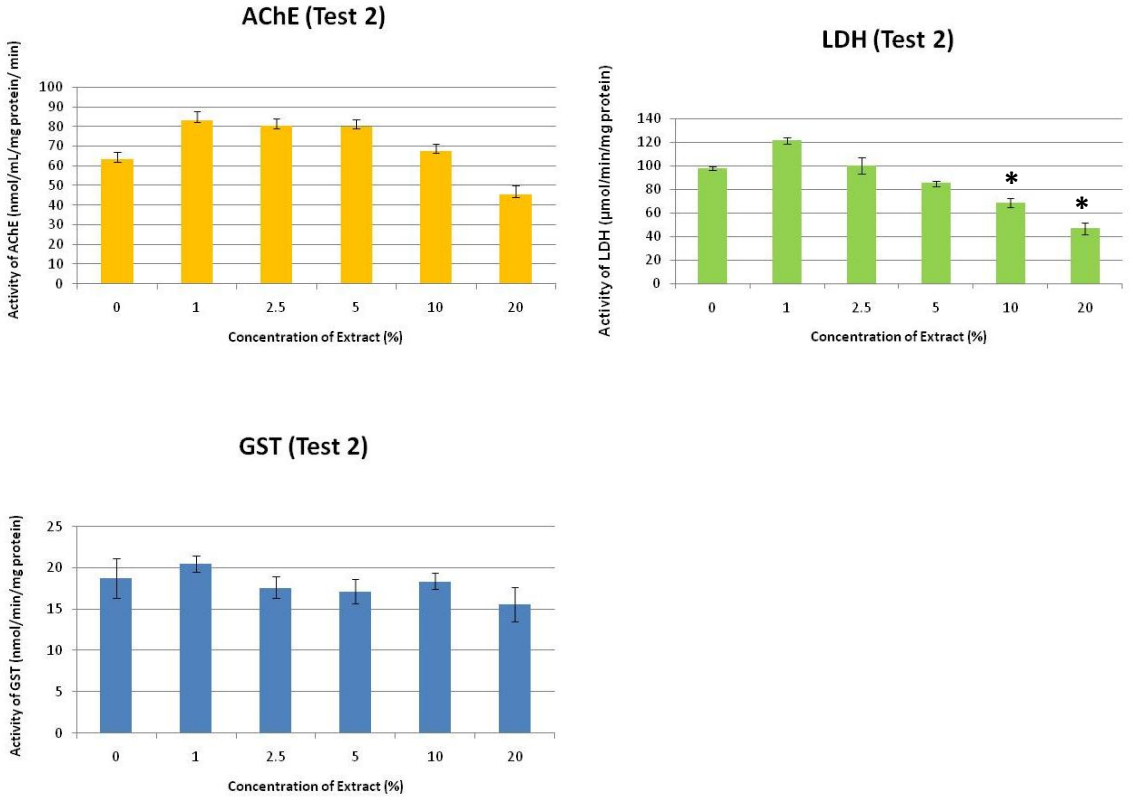

Figure 18: Variation of AChE, LDH and GST activities respectively (mean value ± standard error) on Zebrafish larvae after 96 hours of exposures to water extract, for Test 2. *Asterisks* mean significantly different from control treatment (LDH: Kruskal-Wallis: H=41.910, df=5 (*P*=<0.001)

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Chapter 4 - Final Remarks

The glass system-tested contain titanium dioxide (14.48 mol% TiO_2), phosphorus oxide (42.76 mol% P_2O_5) and calcium oxide (42.76 mol% CaO). The introduction of TiO_2 allows the stabilization of its structure, which is an obstacle concerning this kind of materials (Silva et al., 2008).

In this project is showed an innovative perspective of cytotoxicity tests using two models higher in complexity – from osteoblast resistant cell line (MG-63 cells) to early life stage Zebrafish (*Danio rerio*) whose development is similar between all vertebrates.

Powder glass reactivity has been studied and revealed good results when exposed to solutions with similar composition to human plasma (SBF and PBS) (Silva et al., 2008) and Zebrafish water. It released significant amount of P, Ca and Ti, and others ions when it was immersed in PBS and Zebrafish water. The dissolution on pH was different as expected.
Studies *in vitro* presented good and interesting findings concern to cultures morphology, cells viability and proliferation and expression of genes involved in bone remodeling as referred in Chapter 2.

It was identified a dose-dependent inhibitory effect of MG-63 cells within the concentration 20% to 50% and also time-dependent cumulative deleterious effect between 3 and 7 days. Besides this conclusion, time of cellular events can be adequate for bone building in target area. Reconstruction of bone involved several stages and it is a dynamic process, cells assimilate and integrate, sometimes, signals. The ions dissolved from powder glass are good candidates. Results obtained are examples, because between days 3 and 7 cells recovered (increase in cell growth rate) and convergence of number of viable cells in day 10 for 50% PBS (control) and 50% Extract. Glass formulation should be reformulated with antibiotics and other cooperative molecules in this process such as BMP's. In fact, some ions released from powder glass signalized a good local for osteoblast-like cells attach, slowly when concentration of extract was 50% (day 7, CLSM).

Plaque systems of the material tested showed that less roughness was the most convenient for strong adhesion and spreading of MG-63 cells. This micro-architecture represents the ideal for successful projection of pseudopodes to cells establishment. When plaque was

immersed in SBF any deterioration was detected. There was the formation of a layer rich in Ca and P, an evidence of potential biomineralization and at same time biocompatibility according to good cell's responses. The same precipitate was detected and a stabilized pH value below 7.5 was reported in powder-test, also immersed into SBF (Silva et al., 2008).

PBS was selected as solvent because it is less complex than SBF but this solution is highly saline. For the test in Zebrafish embryos it was necessary a dissolution in Zebrafish water (eggs don't tolerate high salinity levels). In future works toxicity assays will be done with the referred solutions.

The glass composition tested, either in powder or as a plaque, seems to be a promising solution for bone healing processes or even for bone regeneration situations.

Nevertheless, pathways involved are poorly understood. One more complex model was used to tell the history of this bioprocess – embryos of Zebrafish until larvae. It was analyzed one important biomarker involved in development of nervous system and muscles (AChE activity), presence of damage tissue and toxicity by chemical stress (LDH activity) and elimination of pollutants (GST activity) and also the amount of ions Ti, Ca and P into solutions as possible markers of ions dissolved pathways. More experimental data are needed to find principal way(s) of action, perhaps with other model (e.g. *Daphnia magna*) and/or quantification of ions before and after organism exposition. Regarding to AChE test 2, for instance, it was reported a controversial stimulatory effect (in spite of not being statistical different from control). The activity of this enzyme is documented in almost all cases as inhibitory (Domingues et al., 2010). 10% and 20 % of extract exposure in test 2 resulted in chemical stress. In the same test, organism test didn't select GST pathway for toxic elimination. Results of test 1 revealed possible effects of extract.

In future works it would be important to study the expression levels of some genes involved in bone remodeling. For instance, *Dlx* genes below to a family which are associated to pattern and development of the brain, craniofacial structures, and the axial and appendicular skeleton (Kraus et al., 2006). Other study is analyzed phenotypic endpoints during embrionary development of Zebrafish, namely egg coagulation, otolith formation,

eye and body pigmentation, somite formation, heart beat, tail circulation, detachment of the tail-bud from the yolk sac and hatching. After this, other parameters usually studied are oedemas, spine malformation and mortality (Oliveira et al., 2009). Related to cells, extract toxicity screening would be done in primary cells cultures, because they contain different kind of cells; it is more realistic and complex.

Results revealed that this material is promissory in bone regeneration. But more information is needed concerning in way(s) of action interview, because Human being is a very complex and dynamic organism, pools of cells interact at the same time and several pathways appear as answer to stimulus.

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